Functional analysis of activins during mammalian development

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ACTIVINS are dimeric (βAβA; βBβB; βAβB) members of the transforming growth factor-β superfamily[1]. They are widely expressed during murine development[1-6], are highly conserved during vertebrate evolution[1,7-11], and may be involved in mesoderm induction and neurulation in *Xenopus laevis* and *Oryzias latipes*[10-17]. To investigate the function of mammalian activins *in vivo*, we generated mice with mutations either in activin-βA or in both activin-βA and activin-βB. Activin-βA-deficient mice develop to term but die within 24 h of birth. They lack whiskers and lower incisors and have defects in their secondary palates, including cleft palate, demonstrating that activin-βA must have a role during craniofacial development. Mice lacking both activin subunits show the defects of both individual mutants but no additional defects, indicating that there is no functional redundancy between these proteins during embryogenesis. In contrast to observations in lower vertebrates[10-17], zygotic expression of activins is not essential for mesoderm formation in mice.

In the mouse, activin-βA and βB subunits (Fig. 1*a*) are expressed zygotically before implantation[2-4]. After implantation, activin subunit expression is limited to the maternal deciduum until E10.5, when activin-βA begins to be expressed in mesenchymal cells of the developing face, whiskers, hair follicles, heart and digestive tract[5,6,18]. Mice deficient in activin-βB are viable but have defective eyelid development and female reproduction and normal mesoderm formation or neurulation[19]. To investigate the function of activins during mammalian development, mice with a disrupted activin-βA allele were generated using embryonic stem cell technology (Fig. 1*b*). Heterozygous ($act\beta A^{ml}/+$) mice were fertile and viable and were intercrossed to obtain $act\beta A^{ml}/act\beta A^{ml}$ mice (activin-βA-deficient). Genotype analysis of the progeny from these intercrosses (Fig. 1*c*) revealed normal mendelian ratios, with 25.1% wild type (68/271), 49.4% heterozygotes (134/271) and 25.5% homozygotes (69/271), indicating that $act\beta A^{ml}/act\beta A^{ml}$ mice survived to birth. The activin-βA-deficient mice (Fig. 2*a*) appeared to be healthy at birth, breathed normally, and were similar in weight to controls ($act\beta A^{ml}/act\beta A^{ml}$, 1.19 ± 0.12g ($n=22$); littermate controls, 1.24 ± 0.11g ($n=23$)). Although ($act\beta A^{ml}/act\beta A^{ml}$

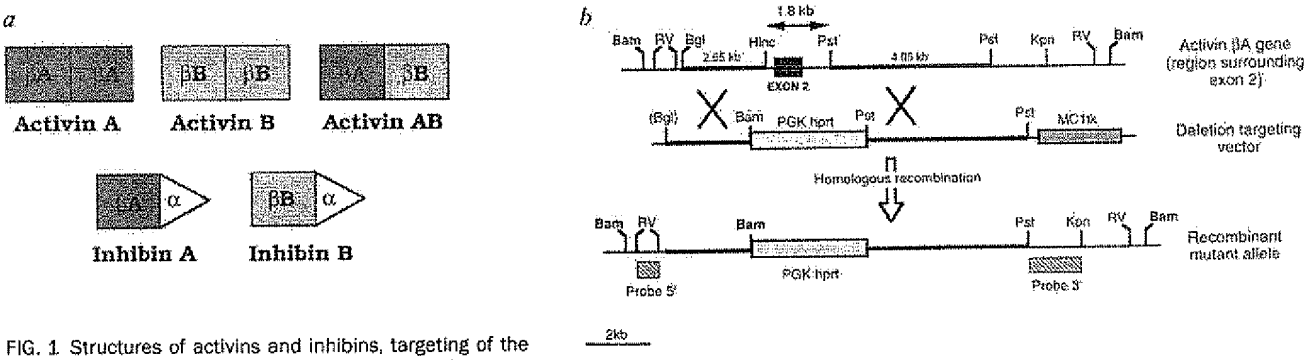

FIG. 1 Structures of activins and inhibins, targeting of the activin-βA gene in embryonic stem (ES) cells, and generation of activin-βA-deficient mice. *a*, Dimeric forms of activins and inhibins. Absence of both βA and βB subunits abolishes all activin and inhibin activity. *b*, The targeting vector to delete exon 2 (1.8 kb of sequence) of the activin-βA gene is shown. The mouse activin-βA gene is a two-exon gene with an 8.8-kb intron. Exon 1 encodes the signal peptide sequence and 101 amino acids of the propeptide; exon 2 encodes 181 amino acids of the propeptide and the 116-amino-acid mature activin-βA peptide. Homologous recombination between this targeting vector and the endogenous mouse activin-βA locus deletes exon 2. The presence of a 4.0-kb fragment using a 5′ probe or a 12.3-kb fragment using a 3′ probe, versus a 14.5-kb wild-type fragment when digested with the restriction endonuclease *Bam*HI is diagnostic of the mutant allele. *c*, Southern blot analysis of DNA from offspring derived from heterozygous matings. Genomic DNA (~5 μg), isolated from the tails of offspring from one litter, was digested with *Bam*HI and analysed using a 5′ probe. The presence of a single 4.0-kb fragment in the four lanes on the left indicates a homozygous mutant (−/−) genotype. The four homozygotes genotyped did not have milk in their stomachs and lacked whiskers.

METHODS. 21.6 kb of isogenic DNA sequence encompassing the 2-exon mouse activin-βA gene was isolated from a mouse 129SvEv library. Linearized vector (25 μg) was electroporated into the *hprt*-negative AB2.1 ES cell line, selected in HAT (where HAT is hypoxanthine, aminopterine, thymidine) and FIAU is (1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil), analysed by Southern blot analysis, and injected into chimaeras as described[30,31]. Enrichment in HAT and FIAU was 14.4-fold compared to HAT alone. Southern blot analysis of ES cell DNA identified 14 targeted clones out of 115 clones screened. The mutant allele was transmitted from chimaeras derived from independent clones βA5-F12 and βA6-D5. To confirm the nature of the mutant allele, a cDNA probe encoding the activin-βA C-terminal (mature) sequence was hybridized to DNA from the $act\beta A^{ml}/act\beta A^{ml}$ mice. The lack of hybridization to a wild-type activin-βA allele confirmed that the mutant $act\beta A^{ml}$ allele was null (data not shown).

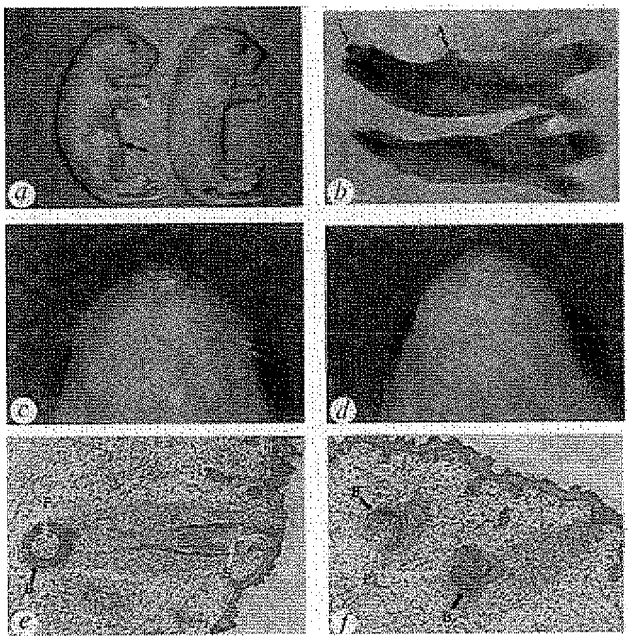

FIG. 2 Morphological and histological analysis of activin-βA mutant and control mice. *a*, Wild-type (left) and activin-βA-deficient homozygote (right) newborn littermate mice. Note the absence of milk in the abdomen of the activin-βA-deficient mouse compared to the wild-type control (arrow). *b*, Medial view of wild-type (top) and $act\beta A^{ml}/act\beta A^{ml}$ (bottom) dissected mandibles stained with alizarin red and alcian blue. The alveolar ridge (A) is the region of the mandible that surrounds the lower molar teeth. The alveolar ridge (A) is less prominent and the incisor (I) is missing in the mutant, consistent with the expression of activin βA in the developing tooth buds[6,18]. There are no primary defects in mandible development. *c* and *d*, Gross analysis of the whisker pads and face of a wild-type control (*c*) and $act\beta A^{ml}/act\beta A^{ml}$ (*d*) newborn mice, showing the absence of whiskers in the mutant (*d*) mouse. *e* and *f*, Histological analysis (haematoxylin and eosin stain[31]) of the whisker follicles of control (*e*) and $act\beta A^{ml}/act\beta A^{ml}$ (*f*) mice; magnification is the same in *e* and *f* and sections are transverse sections through the whisker pads. Note that the distances of the hair papillae (P) from the surface in the mutant (*f*) are shortened compared to the control (*e*). In addition, the hair bulbs (B) in the mutants (*f*) are less well formed than in the control (*e*). The root sheath (S) is obvious as it approaches the epidermis in the control (*e*), but is rarely visible in sections taken from several mutants.

mice progressed to term at the expected frequency, they died within 24 h of birth. The $act\beta A^{ml}/act\beta A^{ml}$ mice lacked whiskers and incisors (Fig. 2*b–d*), consistent with the expression of activin-βA messenger RNA in the mesenchyme of the whisker follicle and teeth primordia[6,18]. Analysis of skeletal preparations showed that the lower incisors were absent (complete absence in 22/25 $act\beta A^{ml}/act\beta A^{ml}$ mice) and that there was a secondary defect in the alveolar ridge of the mandible, the site of formation of the lower molars (Fig. 2*b*). Immature tooth buds could be detected in histological sections (Fig. 3). Histological analysis of the whisker pads revealed that growth and/or differentiation of the whisker follicles was delayed (Fig. 2*e*, *f*). The root sheath, if present, appears to be immature, the cells at the base of the hair bulb often form an irregular pattern, and the hair papilla always lies closer to the surface (Fig. 2*f*); none of these features is evident in control littermate mice. Thus, activin A is required for the normal development of whiskers and teeth.

Newborn $act\beta A^{ml}/act\beta A^{ml}$ mice, identified by their lack of whiskers, failed to suckle because of a cleft palate; 12 of 42 (29%) hybrid background (C57Bl/6/129SvEv) and 3 of 9 (33%) 129SvEv inbred $act\beta A^{ml}/act\beta A^{ml}$ newborn mice had a cleft secondary palate (Fig. 3). Skeletal preparations of 22 $act\beta A^{ml}/act\beta A^{ml}$ newborn mice (hybrid background) that did not have clefts of the secondary palate revealed that 7 of 22 (32%) lacked a hard palate, whereas in most of the remaining mice development of the hard palate was incomplete (Fig. 3).

To generate mice deficient in all activins and therefore lacking both activin-βA and βB subunits (Fig. 1*a*), $act\beta A^{ml}/+$ mice were interbred with $act\beta B^{ml}/+$mice[19] to generate compound heterozygous mutant mice ($act\beta A^{ml}$, $act\beta B^{ml}/+$, +). Compound heterozygous mutant mice were interbred and compound homozygous mutant mice ($act\beta A^{ml}$, $act\beta B^{ml}/act\beta A^{ml}$, $act\beta B^{ml}$) deficient in all activins and inhibins were viable at birth (Fig. 3*f*) and seen at the expected frequency (Table 1). $act\beta A^{ml}$, $act\beta B^{ml}/act\beta A^{ml}$, $act\beta B^{ml}$ mice were readily identifiable as they exhibited phenotypic characteristics of the individual mutant lines. These mice lacked whiskers and incisors (data not shown) and had eyelid defects (namely open eyes; Fig. 3*f*), and appear to have died of palate defects, with three out of nine (33%) having cleft secondary palate. Furthermore, the weight of $act\beta A^{ml}$, $act\beta B^{ml}/act\beta A^{ml}$, $act\beta B^{ml}$ mice was comparable (1.08 ± 0.11 g; $n = 9$) to their littermates (1.08 ± 0.13 g; $n = 35$) and they had no additional defects over those seen in individual activin-βA or βB mutant mice.

The absence of whiskers and incisors and the defects in the formation of the secondary palate in activin-βA-deficient mice

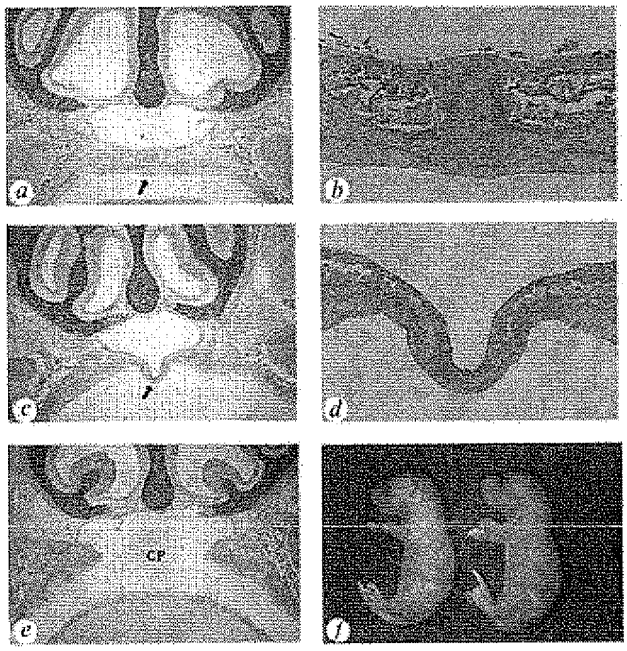

FIG. 3 Histological analysis of the palate and surrounding tissues (*a–e*) and morphological analysis of compound homozygote mice (*f*). Coronal sections at the level of the eye of wild-type (*a*, *b*) and $act\beta A^{ml}/act\beta A^{ml}$ (*c–e*) mice. Note the normal joining of the lateral palatine processes to form the hard and soft palates in the wild-type mouse (arrow) photographed at low (*a*) and high (*b*) magnification. The hard palate (arrow) has not formed in the mutant at low (*c*) and high (*d*) magnification, which is evident from a lack of bone matrix and a sagging of the palate. There is a cleft palate (CP; absence of both hard and soft palates) in another mutant (*e*). *f*, Newborn control (left) and compound homozygote $act\beta A^{ml}$, $act\beta B^{ml}/act\beta A^{ml}$, $act\beta B^{ml}$ (right) mice. Both mice were alive at the time the photograph was taken. The mutant mouse (right) had open eyes and no whiskers and was approximately the same size and weight as the control.

METHODS. Coronal sections through the palates were processed as described[31] and stained with alcian blue and neutral red. Southern blots of DNA from newborn offspring generated by intercrossing of compound heterozygous activin-mutant mice were analysed as described[29,31], using the 5′ probe to determine the activin-βA genotype and a 3′ probe for the activin-βB genotype[19]. Tail DNA was digested with *Bam*HI.

TABLE 1 Genotype analysis of offspring generated from compound heterozygote mice

βA	βB	Number	Observed (%)	Expected (%)
WT*	WT	10	8.0	6.25
WT	+/−	13	10.4	12.5
WT	−/−	10	8.0	6.25
+/−	WT	11	8.8	12.5
+/−	+/−	34	27.2	25
+/−	−/−	16	12.8	12.5
−/−	WT	4	3.2	6.25
−/−	+/−	13	10.4	12.5
−/−	−/−	14	11.2	6.25
Total		125	100.0	100.0

* WT, wild type; +/−, heterozygote; −/−, homozygote.

are consistent with the expression of activin-βA mRNA[6,18], and with the *in vitro* effects of activins on chondrogenesis and bone formation[20,21]. The cleft palate and mandible and incisor defects are also seen in *Msx1*-deficient mice[22]. The incomplete penetrance of the 'cleft' is reminiscent of human cleft palate and the multifactorial nature of its development[23]. Interestingly, mice with the recessive mutation, *oel*, had cleft palate, open eyelids, and died perinatally[24], like compound homozygous activin-βA/βB mutant mice. The phenotype of *oel* mice is consistent with a defect in a common downstream component of both the activin A and B signal transduction cascades. The observation that activin-βA/βB double-mutant mice have a phenotype that is an additive combination of the single mutant phenotypes indicates that individual activin homodimers are not functionally compensating for one another during development and that the activin-βAβB heterodimer does not have a unique function during embryogenesis.

Although we have shown that zygotic activins are not essential for mesoderm induction in mice, experiments with mice carrying a TGF-β1 mutation indicate that maternal TGF-β1 may cross the placenta to partially rescue the mutant mice[25]. As activin-βB-deficient mice have normal mesoderm-derived tissue when delivered from activin-βB-deficient mothers[19], maternal or decidual activin B is not essential; similar studies with activin-βA-deficient females are not possible. Overexpression of activin subunits, receptors and truncated receptors in *Xenopus laevis* and zebra fish could influence mesoderm induction[10–17] as a result of interaction with other TGF-β superfamily members and their receptors[26–28]. For example, truncated *Xenopus* activin type-II receptors (ActRcII or ActRcIIB) block the activity of Vg1, a related TGF-β superfamily member[28]. Identification of the serine/threonine kinase receptors that are expressed at or around the time of mesoderm induction will give us a better understanding of the TGF-β-related proteins involved in this process. Lastly, the phenotype of mutant mice deficient in one of the activin receptors, ActRcII (see accompanying Letter[29]), overlaps minimally with that of activin-deficient mice. This calls into question the relevance of ActRcII as a receptor for activin A and/or activin B during embryogenesis. Alternatively, the other type-II activin receptor, ActRcIIB, might be important in activin signal transduction during embryogenesis. A full understanding of the specificity of these ligand/receptor interactions must come from analysis of ActRcIIB mutant mice and the demonstration of epistasis in compound mutant ligand/receptor mice. □

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Different phenotypes for mice deficient in either activins or activin receptor type II

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ACTIVINS are believed to initiate a signal transduction cascade by binding to serine/threonine kinase receptors types I and II[1–3]. Activins[4] bind to several different receptors *in vitro*[1–3], but the significance of this interaction *in vivo* has not been confirmed. To test the function of the type II activin receptor (ActRcII) in mammalian development and reproduction, we generated a null mutation in the ActRcII gene in mice using embryonic stem cell technology. We expected ActRcII-deficient mice to phenocopy activin-deficient mice. A few ActRcII-deficient mice had skeletal and facial abnormalities reminiscent of the Pierre–Robin syndrome in humans[5,6], but most lacked these defects and developed into adults; their follicle-stimulating hormone was suppressed, and their reproductive performance was defective. These findings confirm a role of ActRcII in activin signalling in pituitary gonadotrophs. The striking lack of overlap between phenotypes of ActRcII-deficient and activin-deficient mice suggests that the ligands that signal through ActRcII during embryonic development are not activins.

We tested the function of ActRcII in activin signalling by mutating the ActRcII gene using ES cell technology to delete exon 1, which includes both the initiation codon and the signal peptide sequence (Fig. 1*a*). Mice heterozygous for this null allele ($actRcII^{ml}/+$) were intercrossed to produce homozygote F_2 ActRcII-deficient ($actRcII^{ml}/actRcII^{ml}$) mice (Fig. 1*b*, *c*). ActRcII-deficient mice would be expected to be a phenocopy of the activin-βA, activin-βB or activin-βA/βB double-mutant mice (that is, to exhibit defects in eyelid development, lack whiskers